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Norepinephrine Augments *Salmonella enterica*-Induced Enteritis in a Manner Associated with Increased Net Replication but Independent of the Putative Adrenergic Sensor Kinases QseC and QseE[▽]

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Stress has long been correlated with susceptibility to microbial infection. One explanation for this phenomenon is the ability of pathogens to sense and respond to host stress-related catecholamines, such as norepinephrine (NE). In Gram-negative enteric pathogens, it has been proposed that NE may facilitate growth by mediating iron supply, or it may alter gene expression by activating adrenergic sensor kinases. The aim of this work was to investigate the relative importance of these processes in a model in which NE alters the outcome of *Salmonella enterica* serovar Typhimurium infection. A bovine ligated ileal loop model was used to study the effect of NE on enteritis induced by *S. Typhimurium* and on the bacterial in vivo replication rate. Mutants lacking putative adrenergic receptor genes were assessed in the loop model, in a calf intestinal colonization model, and in vitro. *S. Typhimurium*-induced enteritis was significantly enhanced by addition of 5 mM NE. This effect was associated with increased net bacterial replication in the same model. Exogenous ferric iron also stimulated bacterial replication in the medium used but not transcription of enteritis-associated loci. The putative adrenergic sensors QseC and QseE were not required for NE-enhanced enteritis, intestinal colonization of calves, or NE-dependent growth in iron-restricted medium and did not influence expression or secretion of enteritis-associated virulence factors. Our findings support a role for stress-related catecholamines in modulating the virulence of enteric bacterial pathogens in vivo but suggest that bacterial adrenergic sensors may not be the vital link in such interkingdom signaling in *Salmonella*.

Physiological stress affects the human intestinal microbiota and may play a role in primary intestinal mucosal diseases, such as inflammatory bowel syndrome, and systemic inflammatory states (1). Similarly, in food-producing animals, stress associated with social interaction, handling, and transport has been correlated with increased excretion of the zoonotic diarrheal pathogens *Escherichia coli* and *Salmonella enterica* (43). A key response of the enteric nervous system to stress is the release of norepinephrine (NE). In mice subject to partial hepatectomy, it was found that NE reaches the intestinal lumen and modulates the virulence of *Pseudomonas aeruginosa* (2), which is of relevance to the high rate of gut-derived sepsis caused by this pathogen in surgical patients.

Though evidence exists that stress-associated catecholamines exert their effects partly by acting on host tissues (8, 18), it is clear that they also act directly on bacteria promoting growth (29) and virulence gene expression (28, 41) in enterohemorrhagic *E. coli* (EHEC) O157:H7. Two major nonexclusive hypotheses have been put forward to explain these effects. The first posits that NE and related catecholamines facilitate the supply of iron to Gram-negative bacteria under iron-limiting conditions (16, 17). As many bacterial virulence genes are

growth phase regulated, this may explain downstream effects. The second hypothesis posits that induction of virulence gene expression results from microbial detection of catecholamines via specific receptors (30, 41, 42). For example, the presence of a non- α , non- β , primitive adrenergic receptor was proposed (30).

Recently, an adrenergic receptor was identified in EHEC O157:H7 (10, 24). This arose from the finding that the *qseBC* genes are required for epinephrine to stimulate motility (41, 42). QseBC exhibits homology to two-component systems, which typically comprise a sensor kinase and cognate response regulator. It was subsequently proven that QseC binds NE, autophosphorylates in response to epinephrine and NE, and transfers the phosphate moiety to QseB, thereby modulating the expression of genes under its control (10). However, some enteric pathogens respond to NE but lack QseC (e.g., *Campylobacter jejuni*), and this may reflect the presence of other catecholamine receptors or alternative mechanisms. It was reported recently that another two-component system (QseEF) acts as a secondary receptor for epinephrine in EHEC O157:H7 (38, 39).

S. enterica serovar Typhimurium encodes orthologues of EHEC O157:H7 *qseBC* and *qseEF*. Recent studies have indicated that *S. Typhimurium* QseBC influence systemic virulence in mice (31), intestinal colonization of swine, and NE-induced motility (3). Furthermore, an inhibitor of EHEC O157:H7 QseC signaling, LED209, reportedly aids control of systemic salmonellosis in mice (37). However, two-component systems

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TABLE 1. Primers used in this study

Primer function and name	Sequence (5'–3')
Mutagenesis	
QseC-For	CGCACCCTGCACGGCATCGG CTACACCCTGGGTGACGCA TGGTGTAGGCTGGAGCTG CTTC
QseC-Rev	CGTTTTTGGCAAAAGTCTCTG TTACCAACTTACTACGGCCT CCATATGAATATCCTCCTTA
QseE-For	GCTTCCCTCGGTTAGCATCTT TTTTATTCTCTTTTATGTG GTGTAGGCTGGAGCTG CTTC
QseE-Rev	CGCACCAGGCTTAAATTCATA TTAGTGTGTTGTCAGATGCA GGCATATGAATATCCTC CTTA
Confirmation of mutations by PCR	
k1	CAGTCATAGCCGAATAGCCT
k2	CGGTGCCCTGAATGAAGTGC
QseC-Up	TGCAGTGGAAAGTCCATGTT
QseC-Down	CCCCGTTGATAATCAGGATG
QseE-Up	TAGCCCGGCTTTGTTGTATC
QseE-Down	TGGACGAAAACGTGTGACAT
Probe amplification	
QseC-P1	CAACGTCTCAGCCTGCGC
QseC-P2	CTACGGCCTCAAATCCACC
QseE-P1	GTGTTTGTGTCAGATGCAGGC
QseE-P2	GTGAGCGACACGTTGAAG

are known to integrate multiple signals (QseC detects epinephrine, NE, and autoinducer-3 [10]; QseE detects epinephrine, sulfate, and phosphate) (38). Hence, it remains to be proven that QseC is required for NE-induced events in vivo. We have previously reported that NE augments *E. coli* O157:H7-induced enteritis and mucosal adherence in a bovine ligated loop model (44). Here, we showed that the effect of NE is not restricted to EHEC and sought to evaluate the relative importance of adrenergic sensors versus net replication in the enhancement of enteritis by norepinephrine.

MATERIALS AND METHODS

Bacterial strains and plasmids. A spontaneous nalidixic acid-resistant derivative of the bovine *S. Typhimurium* isolate ST4/74 was used and was fully virulent in calves (33). Strains were cultured in Luria-Bertani (LB) medium or serum-SAPI medium (28). Antibiotics were used at the following concentrations: nalidixic acid (Nal; 20 µg ml⁻¹), ampicillin (Amp; 100 µg ml⁻¹), and kanamycin (Kan; 50 µg ml⁻¹). Plasmids pCP20 (9), pKD4, and pKD46 (12) were obtained from the *E. coli* Genetic Stock Centre, Yale University. Plasmid pHSG422 (20) was electroporated into *S. Typhimurium* strains at 25°C. *S. Typhimurium* SL1344 derivatives containing single-copy green fluorescent protein (GFP) transcriptional fusions were obtained from J. Hinton (Institute of Food Research, Norwich, United Kingdom). Strain SL1344 is a *hisG* derivative of ST4/74 (22). The strains contained the following transcriptional fusions: JH3008 (a promoterless *gfp*⁺), JH3009 (*ssaG-gfp*⁺), JH3010 (*prgH-gfp*⁺), and JH3016 (*rpsM-gfp*⁺) (21).
General molecular techniques. GoTaq DNA polymerase and restriction enzymes were purchased from Promega Corporation (Southampton, United Kingdom) and used according to the manufacturer's recommendations. Oligonucleotide primers were obtained from Sigma Genosys (Poole, United Kingdom) (Table 1). Genomic DNA from *Salmonella* was prepared by cetyltrimethyl ammonium bromide extraction as described previously (36). DNA probes for Southern hybridization consisted of digoxigenin-labeled PCR products amplified from

strain ST4/74 with a digoxigenin DNA labeling and detection kit supplied by Roche Molecular Biochemicals (Mannheim, Germany).
Analysis of virulence gene expression and motility. The reporter strains JH3008, JH3009, JH3010, and JH3016 and their derivatives were grown overnight in LB medium at 37°C with agitation and subcultured 1:50 into serum-SAPI medium with or without 5 mM NE. These were incubated at 37°C at 130 rpm for 16 h, and samples were taken for measurement of fluorescence as described previously (5). The effect of 25 µM Fe(III) nitrate was similarly assessed. Secreted proteins were purified and analyzed as described previously (23). Test cultures were supplemented with 100 µM NE, and controls were supplemented with an equivalent volume of diluent. Motility assays were performed in Dulbecco's modified Eagle's medium (DMEM) containing 0.3% (wt/vol) agar as described previously (31).
Construction of sensor kinase mutants. *S. Typhimurium* orthologues of the EHEC O157:H7 adrenergic sensor genes *qseC* and *qseE* were mutated by λRed recombinase-mediated integration of linear PCR products. Primers were designed to amplify the pKD4-encoded Kan resistance cassette, including approximately 40-bp extensions from the 5' and 3' ends of the predicted *qseC* (also known as *preB* or STM3178 in *S. Typhimurium*) and *qseE* (also known as STM2564) genes (Table 1). PCR products were purified and electroporated into ST4/74 (pKD46) induced to express λRed recombinase as described previously (12). Kan^r recombinants were selected and cured of pKD46 by culture at 37°C. Mutations were confirmed by PCR with primers specific to the Kan cassette (k1 and k2) and primers flanking the target genes (Table 1). To remove the Kan cassette from the *ΔqseC*::Kan and *ΔqseE*::Kan mutants, plasmid pCP20 was introduced, and flippase recombinase was induced as described previously (12). These mutants, *ΔqseC* and *ΔqseE*, were confirmed by PCR using the flanking primers. A double mutant, *ΔqseC ΔqseE*::Kan was prepared from *ΔqseC* by a second round of λRed mutagenesis, and the Kan cassette was excised to produce *ΔqseCE*. Sequencing of amplicons confirmed the expected deletions (data not shown), and all mutants were further verified by Southern blotting of HindIII- or KpnI-digested genomic DNA using gene-specific probes (Fig. 1). The *ΔqseC*, *ΔqseE*, and *ΔqseCE* mutants exhibited similar growth kinetics to ST4/74 in LB medium, M9 minimal medium, and DMEM (data not shown). The *ΔqseC*::Kan and *ΔqseE*::Kan mutations were also transduced into the GFP reporter strains, JH3008, JH3009, JH3010, and JH3016, using P22 *int* phage to evaluate their effect on transcription of reporter fusions.
Experimental animals. Animal experiments were conducted according to the Animal (Scientific Procedures) Act 1986 (license 30/2485) with the approval of

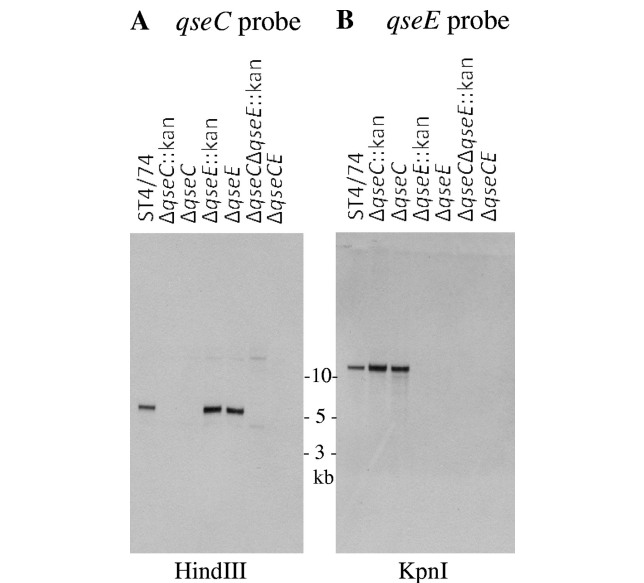


FIG. 1. Verification of *S. Typhimurium* sensor kinase mutants by Southern blot analysis. Genomic DNA from the indicated strains was digested with HindIII or KpnI and hybridized to sensor kinase gene probes, as follows: a *qseC* probe amplified using primers QseC-P1 and QseC-P2 (A) or a *qseE* probe amplified using primers QseE-P1 and QseE-P2 (B) (Table 1).

the local Ethical Review Committee. Friesian bull calves were confirmed to be *Salmonella* negative as described previously (35).

Ligated loop assay for quantification of enteropathogenesis. The model is described in detail elsewhere (45). Briefly, calves (approximately 28 days old) were maintained under terminal general anesthesia, a laparotomy was performed, and ligated loops were constructed in the mid ileum. Bacteria were grown in serum-SAPI medium at 37°C overnight. Viable counts of the inocula were determined by retrospective plating of serial dilutions. Immediately prior to loop inoculation, a 0.5 M stock of L-norepinephrine (bitartrate salt; Sigma, Poole, United Kingdom) was prepared in phosphate-buffered saline. Bacterial cultures were divided into two samples and were supplemented with 5 mM NE or with an equivalent volume of diluent. Five-milliliter volumes were injected into triplicate loops in a semirandomized order in each animal. Polymorphonuclear cells (PMNs) were isolated from 100 ml of venous blood, labeled with ^{111}In oxinate, and reinjected into the jugular vein. Twelve hours after inoculation, enteropathogenesis was quantified by measuring fluid accumulation as a ratio of loop length (V/L , where V is volume in ml and L is length in cm), and infiltration of ^{111}In -labeled neutrophils was determined relative to control loops.

Determination of the effect of NE on net replication in vivo. Calf ileal loops were constructed as above. *S. Typhimurium* strains containing the temperature-sensitive plasmid pHSG422 were cultured on LB agar containing Nal, Amp, and Kan (Nal-Amp-Kan) at 30°C. Several colonies were inoculated into serum-SAPI medium, cultured overnight at 25°C, subcultured 1:3 into serum-SAPI medium, and incubated for 2 h at 37°C to reduce plasmid copy number to approximately 1. Cultures with or without 5 mM NE were then prepared. Viable counts of the inocula were determined on MacConkey agar containing Nal-Amp-Kan (for enumeration of pHSG422-containing bacteria) and Nal alone (for total counts) immediately before loop inoculation. Twelve hours after inoculation, triplicate 1-g ileal mucosa biopsies were cut from each loop, homogenized, serially diluted, plated as for the inocula, and cultured at 30°C.

Determination of the effect of NE and ferric iron on net replication in vitro. ST4/74 (pHSG422) was cultured on LB agar containing Nal-Amp-Kan at 30°C. Several colonies were inoculated into serum-SAPI medium, cultured overnight at 25°C, subcultured 1:3 into serum-SAPI medium, and incubated for 2 h at 37°C to reduce plasmid copy number to approximately 1. Cultures with no addition, with 5 mM NE, and with 25 μM Fe(III) nitrate were then prepared. These were incubated at 37°C statically for 12 h to mimic the loop experiments. Viable counts of the inocula and the final cultures were determined on LB agar containing Nal-Amp-Kan (for enumeration of pHSG422-containing bacteria) and Nal alone (for total counts). These plates were incubated overnight at 30°C.

Determination of competitive indices (CIs) in calves. Bacteria were grown in LB broth overnight at 37°C. Wild-type (WT) and mutant strains were mixed in equal numbers (ca. 1×10^8 CFU per calf) in 20 ml of antacid (34) and used to inoculate calves orally. Fecal samples were collected daily for up to 5 days. Wild-type and mutant bacteria were enumerated in feces and tissue samples collected at postmortem, and CIs were calculated as described previously (36).

Effect of catecholamines on growth. Overnight LB cultures of wild-type and mutant strains were diluted in serum-SAPI medium with or without catecholamines (all at 100 μM) (Sigma, Poole, United Kingdom) as indicated in the Fig. 6 legend. Viable inocula were determined as described previously (15). Cultures were incubated statically at 37°C in a humidified, 5% CO_2 atmosphere for 18 h, and final counts were determined by serial dilution and plating.

Statistical analysis. For analysis of competitive indices, a Mann-Whitney non-parametric test was used to determine whether the output ratio of mutant to parent strain differed significantly from the input ratio. The data from bovine ligated loop experiments and from the in vitro experiments were analyzed using a Student *t* test. *P* values of <0.05 were considered significant.

RESULTS

Norepinephrine enhances *S. Typhimurium*-induced enteritis. Previous results from our laboratory indicated that ST4/74 cultured in rich medium induced significant secretory and inflammatory responses in the bovine ligated ileal loop model (33, 46). In contrast, we found that ST4/74 grown in the iron-restricted serum-SAPI medium did not induce a significant increase in either fluid secretion or PMN influx (Fig. 2). However, the addition of 5 mM NE led to a significant induction in both fluid accumulation (Fig. 2A) ($P < 0.005$) and neutrophil influx (Fig. 2B) ($P < 0.05$) relative to ST4/74 with diluent.

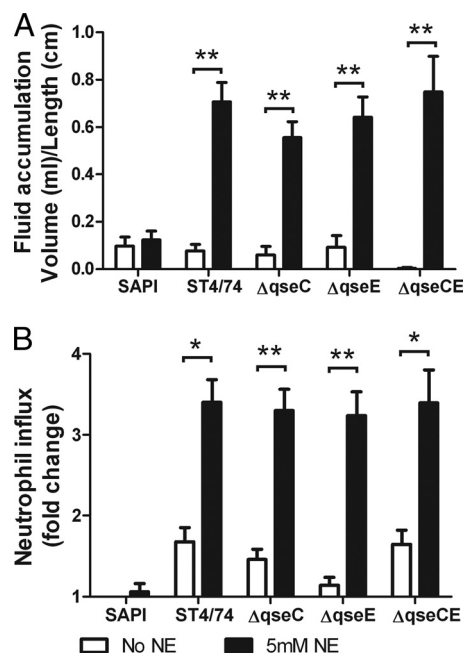


FIG. 2. Effect of 5 mM NE on intestinal secretory and inflammatory responses induced by ST4/74 and mutant strains in bovine ligated ileal loops. The dose per loop was ca. 5×10^9 CFU. (A) Fluid accumulation. The ratio of the volume of fluid accumulated to loop length for each treatment was determined from triplicate loops in each calf. The values shown represent the means and standard errors of the means (SEMs) of the results from six calves for serum-SAPI medium and ST4/74, four calves for $\Delta qseC$ and $\Delta qseE$, and two calves for $\Delta qseCE$. (B) Neutrophil influx. Total ^{111}In activity in the contents and mucosa was corrected for loop length and expressed as a ratio of the total activity in loops containing SAPI medium alone. The values shown represent the means and SEMs of the results from four calves for SAPI medium and ST4/74 and two calves for the mutants, with triplicate loops for each treatment in each calf. The results were analyzed using a two-tailed Student *t* test. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

Addition of NE to serum-SAPI medium did not significantly increase either fluid accumulation or neutrophil influx, indicating that the enhanced enteritis observed with ST4/74 plus NE was unlikely to be due to the effect of NE on host tissues alone. The NE effect, coupled with the ability to evaluate multiple treatments relative to controls in the absence of inter-animal variation, indicated that the loop model was suitable to assess the relative importance of sensor kinases and net replication.

QseC and QseE are not required for NE-enhanced *S. Typhimurium*-induced enteritis. The involvement of QseC and QseE in NE enhancement of *S. Typhimurium*-induced enteritis was investigated as above using defined mutants. The $\Delta qseC$, $\Delta qseE$, and $\Delta qseCE$ mutants all elicited a significant increase in fluid secretion in the presence of 5 mM NE relative to controls ($P < 0.005$) (Fig. 2A). Similarly, the three mutants induced a significant neutrophil influx in the presence of NE (Fig. 2B) ($P < 0.005$ for the single mutants and $P < 0.05$ for the double mutant). Importantly, the elevated secretory and inflammatory responses were comparable to those observed with the wild type (Fig. 2).

NE does not induce expression of type III secretion system (T3SS) genes or motility in vitro. T3SS-1 (46), T3SS-2 (4, 11), and flagella (40, 48) are known to play key roles in *S. Typhimurium*-induced enteritis in the bovine loop model. Furthermore, it has been reported that NE activates a T3SS in *E. coli* O157:H7 (41). We therefore investigated whether NE alters expression of T3SS-1, T3SS-2, or motility in *S. Typhimurium*.

Overnight cultures of the *S. Typhimurium* GFP reporter strains JH3008, JH3009, JH3010, and JH3016 with or without transduced mutations in the sensor kinase genes were subcultured into serum-SAPI medium in the presence or absence of 5 mM NE, and growth continued for 16 h at 37°C. These conditions were similar to those used in the ligated loop experiments (Fig. 2; see also Fig. 4). The transcription level of the T3SS-1 gene *prgH* was very low under the conditions tested and not significantly altered by NE (Fig. 3A). Interestingly, expression of the T3SS-2 gene *ssaG* was significantly reduced by addition of 5 mM NE to JH3009, JH3009 Δ *qseC*, and JH3009 Δ *qseE*. Transcription of the *rpsM* housekeeping gene was elevated in the presence of NE, though not significantly so.

Addition of NE or mutation of *qseC*, *qseE*, or both genes had no clear effect on the levels of secreted proteins produced by ST4/74 under T3SS-1-inducing conditions, including on the known T3SS-1 proteins SipA and SipC (Fig. 3B). The position of bands corresponding to SipA and SipC is known from peptide sequencing and Western blotting with specific antisera (data not shown). In contrast to previous studies, inclusion of NE did not consistently affect the motility of either wild-type or the *qse* mutants (Fig. 3C). Similarly, mutation of *qseC* had no effect on motility. However, mutation of *qseE* or both sensor kinase genes produced a modest but statistically significant reduction in motility (Fig. 3C) ($P < 0.05$).

NE enhances in vivo net replication of *S. Typhimurium*. We previously showed that the greater enteric virulence of *S. Typhimurium* in pigs relative to the host-restricted *S. enterica* serovar Choleraesuis was associated with its higher net replication rate as determined by analysis of plasmid partitioning (34). In this model, enhanced net replication of *S. Typhimurium* was evident within 12 h after loop inoculation and was correlated with induction of proinflammatory cytokine mRNAs and neutrophil recruitment (34). We therefore investigated if the enhancement of *S. Typhimurium*-induced enteritis by NE was associated with increased net replication. For this, bovine ileal loops were inoculated with pHSG422-bearing *S. Typhimurium* derivatives. Plasmid pHSG422 is unable to replicate at body temperature; therefore, a fast-growing organism will yield a higher number of total bacteria, fewer of which will be plasmid bearing, whereas a slower-growing organism will reach lower numbers, but more of the population will retain the plasmid (19).

The replication of ST4/74, Δ *qseC*, and Δ *qseCE* (containing pHSG422) was examined in triplicate loops in two calves in the presence or absence of 5 mM NE. Twelve hours after inoculation, the total number of bacteria associated with the ileal mucosa was enumerated, and the proportion maintaining pHSG422 was determined. The results for the wild-type showed that bacterial recovery was significantly increased in the presence of NE (Fig. 4A) ($P < 0.05$), while the proportion

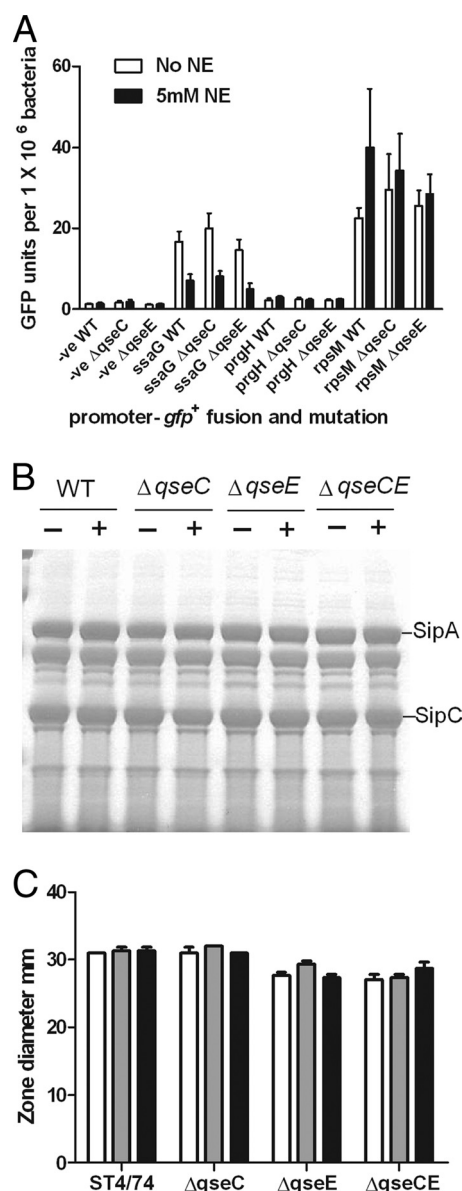


FIG. 3. Effect of NE on expression of T3SS-1, T3SS-2, and motility genes. (A) GFP reporter strains were grown in serum-SAPI medium with or without 5 mM NE for 16 h, and gene expression was measured by GFP fluorescence intensity. The GFP gene fusions were JH3008 carrying a promoterless *gfp*⁺ gene, JH3009 harboring *ssaG* (a T3SS-2 gene fused to *gfp*⁺); JH3009 harboring *prgH* (a T3SS-1 gene fused to *gfp*⁺), and JH3016 harboring *rpsM* (a constitutively expressed gene fused to *gfp*⁺). The graph shows the means and SEMs from three separate experiments, with two fluorescence readings for each. (B) Coomassie blue-stained gel of secreted proteins from the ST4/74 wild type and Δ *qseC*, Δ *qseE*, and Δ *qseCE* mutants grown for 4 h at 37°C in the presence (+) or absence (–) of 100 μ M NE. The positions of SipA and SipC are indicated and were revealed by peptide sequencing of excised bands (data not shown) and Western blotting with specific antisera for SipA (27) and SipC (34). (C) Motility of ST4/74 and Δ *qseC*, Δ *qseE*, and Δ *qseCE* mutants with no NE (white bars), 5 μ M NE (gray bars), and 50 μ M NE (black bars). Bars represent means and SEMs ($n = 4$).

of bacteria containing the plasmid was reduced (Fig. 4B) ($P < 0.05$).

Similar to the wild type, the Δ *qseC* and Δ *qseCE* mutants also replicated significantly faster in vivo in the presence of NE

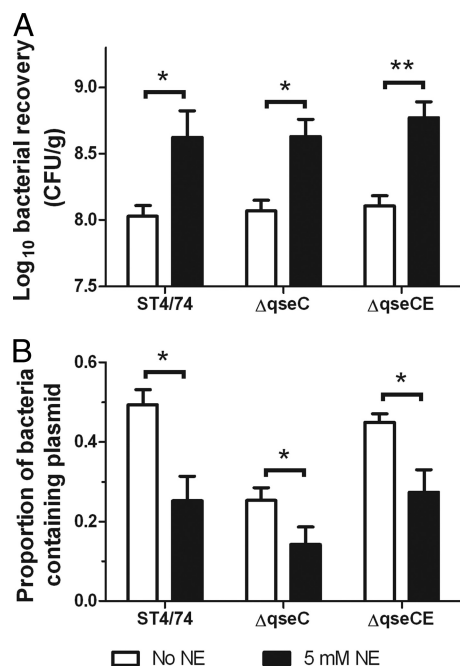


FIG. 4. Effect of 5 mM NE on in vivo net replication of *S. Typhimurium*. Ligated ileal loops were injected with ST4/74, $\Delta qseC$, and $\Delta qseCE$, each containing the pHSG422 plasmid, with or without 5 mM NE. Triplicate loops per calf (two calves) were injected for each treatment. The doses were 3.19×10^9 CFU per loop for ST4/74, 3.2×10^9 CFU for $\Delta qseC$, and 3.16×10^9 CFU for $\Delta qseCE$. The proportions of bacteria containing the plasmid in these three inocula were 0.54, 0.47, and 0.48, respectively. Addition of NE to the inocula did not significantly alter the bacterial numbers or proportions before injection into ileal loops in either experiment. (A) Total bacterial recovery, represented as log₁₀ mean CFU/g of tissue and SEM. (B) Proportion of recovered bacteria containing pHSG422. Bars represent mean proportions and SEMs. The effect of NE was analyzed using a two-tailed Student *t* test. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.005$.

(Fig. 4). Total cell densities were significantly elevated upon treatment with NE (Fig. 4A) ($P < 0.05$ for $\Delta qseC$ and $P < 0.005$ for $\Delta qseCE$) while the proportions of plasmid-bearing bacteria were significantly reduced ($P < 0.05$). Thus, NE enhances net replication of *S. Typhimurium* in the same model and time frame as it augments enteritis and does so independently of putative adrenergic sensor kinases.

One possible explanation for the increased in vivo net replication in the presence of NE is its ability to supply iron from transferrin to the bacteria. We therefore investigated the effect of ferric iron on bacterial replication in vitro under conditions that mimic those used for the in vivo net replication experiments. The final bacterial numbers achieved by ST4/74 (pHSG422) after 12 h of growth in serum-SAPI medium from a high starting bacterial density were significantly increased by the addition of 25 μ M Fe(III) nitrate ($P < 0.05$) (Fig. 5A) relative to cultures with no addition, and the proportion of bacteria retaining the plasmid was reduced although this was not statistically significant (Fig. 5B). The effect of 25 μ M ferric iron was similar but more marked than the effect of 5 mM NE in these experiments.

Since iron is known to induce T3SS-1 gene expression under

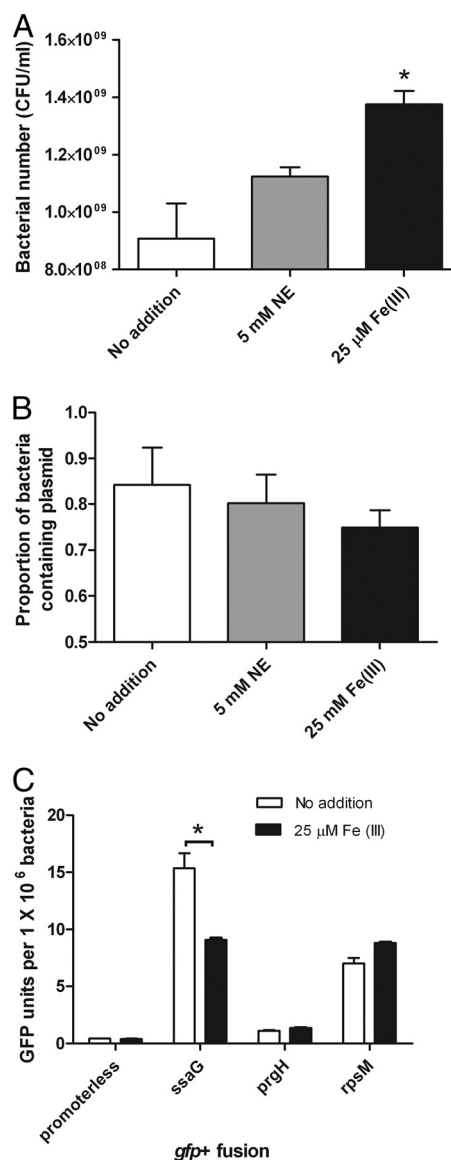


FIG. 5. The effect of ferric iron on *S. Typhimurium* replication and virulence gene expression in serum-SAPI medium. The in vitro net replication of ST4/74 (pHSG422) in serum-SAPI medium for 12 h was determined in cultures with no addition, 5 mM NE, or 25 μ M Fe(III) nitrate. (A) Final bacterial numbers. Bars represent means and SEM ($n = 11$). (B) Proportion of bacteria containing the temperature-sensitive plasmid pHSG422. Bars represent means and SEM ($n = 11$). (C) The effect of 25 μ M Fe(III) nitrate on expression of T3SS-1 and T3SS-2 after growth for 16 h in serum-SAPI medium. The graph shows the means and SEMs from two experiments, with two fluorescence readings for each.

some conditions (13), the effect of 25 μ M Fe(III) nitrate was assessed in serum-SAPI medium using the *gfp*⁺ reporter strains. The expression of the T3SS-1 gene *prgH* was not induced after 16 h in serum-SAPI medium (Fig. 5C) and remained very low. Expression of the T3SS-2 gene *ssaG* was significantly reduced by ferric iron ($P < 0.05$) (Fig. 5C). The effect of iron in this medium was similar to the effect of NE (Fig. 3A).

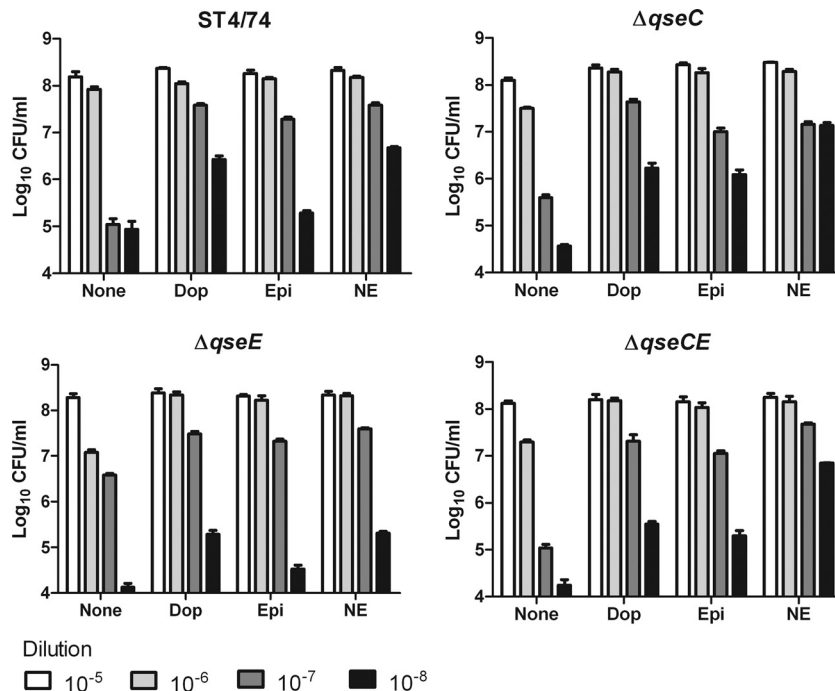


FIG. 6. Effect of catecholamines on growth of ST4/74 and *ΔqseC*, *ΔqseE*, and *ΔqseCE* mutants. Overnight cultures of the indicated strains were diluted as shown in the legend into serum-SAPI medium, with or without catecholamines at 100 μM, and grown for 18 h statically at 37°C. Bacteria were enumerated by serial dilutions and plating. None, no addition; Dop, dopamine; Epi, epinephrine. Values represent the means and standard deviations of triplicate platings from duplicate cultures.

QseC and QseE are not required for catecholamine-dependent growth in serum-SAPI medium. ST4/74 grew poorly in serum-SAPI medium from a low starting density, attaining just ca. 10⁵ CFU/ml 18 h after dilution to 10⁻⁷ or 10⁻⁸ (Fig. 6). However, growth was induced by inclusion of NE, dopamine, or epinephrine. Epinephrine, which is not produced in the gut (14) was the least effective catecholamine at promoting growth. The *ΔqseC*, *ΔqseE*, and *ΔqseCE* mutants all exhibited an ability similar to that of the wild type to respond to the catecholamines. These in vitro growth results confirmed that the sensor kinases play no role in *Salmonella* growth responses to catecholamines.

QseC and QseE are not required for intestinal colonization of *S. Typhimurium* in calves. To assess the role of QseC and QseE in intestinal colonization, competitive indices were derived after coinfection of calves with *ΔqseC*::Kan or *ΔqseC ΔqseE*::Kan relative to the wild-type (Fig. 7). Although some of the fecal samples contained less of the mutant than the parent, particularly for the double mutant, the output ratios were not significantly different from the input ratios owing to the extent of variability. At most of the intestinal sites, the mutants did not colonize significantly less than the wild type. The only exception was that the *qseC* mutant was recovered in signifi-

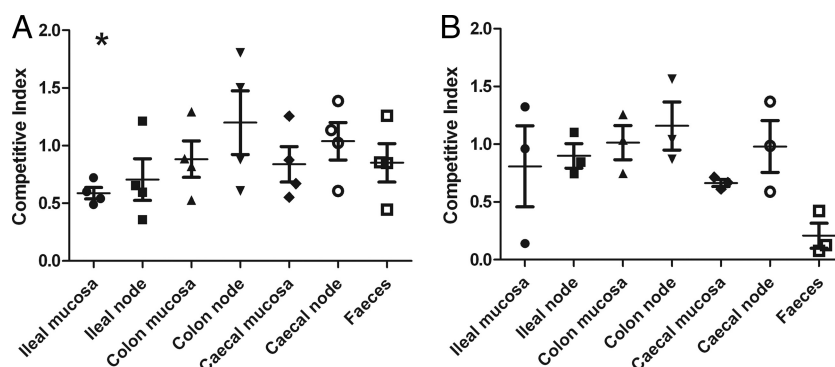


FIG. 7. The *S. Typhimurium* *qseC* and *qseE* genes are not essential for bovine intestinal colonization. ST4/74 and mutant strains were coinoculated orally into calves. (A) WT versus *ΔqseC*::Kan mutant (n = 4). (B) WT versus *ΔqseC ΔqseE*::Kan mutant (n = 3). CIs were determined in samples at 5 days postinfection (except for two calves with the double mutant, which reached their humane endpoints at day 4). The CI was calculated as output ratio (mutant/WT) divided by input ratio (mutant/WT). The mean (horizontal line) and SEM at each site are shown. Output and input ratios were compared using a Mann Whitney test. A P value of <0.05 was taken as significant (indicated by an asterisk).

cantly fewer numbers than the wild type from the ileal mucosa ($P = 0.027$).

DISCUSSION

While the molecular mechanisms by which bacteria, particularly *S. enterica* and *E. coli*, respond to stress-related catecholamines have been the subject of considerable research in vitro, there have been few studies using animal models to assess the relevance of proposed mechanisms for catecholamine-enhanced virulence. Intra-gastric administration of NE to mice a day before infection with *S. Typhimurium* enhanced cecal colonization and systemic spread (32, 47). This effect was blocked by simultaneous mutation of three catecholate siderophore receptor genes, *fepA*, *iroN*, and *cir*, indicating that the effect may involve NE-mediated iron supply (32, 47). Further, chicks given NE twice daily by crop instillation exhibited elevated levels of *S. enterica* serovar Enteritidis in the ceca and liver compared to controls (32).

Although evidence exists that EHEC O157:H7 binds epinephrine and NE via adrenergic sensors, modulating the expression of genes under the control of cognate response regulators (10), it is not clear whether this is vital for hormone-induced changes in pathology. QseC is required for full virulence of EHEC O157:H7 in infant rabbits (10) and influences *S. Typhimurium* virulence in swine (3) and, to a lesser degree, mice (31); however, it cannot be concluded that this reflects the importance of interkingdom signaling since two-component systems integrate multiple signals and the relative importance of catecholamine sensing has not been directly established.

Using a bovine ligated ileal loop model, we previously showed that NE augments *E. coli* O157:H7-induced enteritis and adherence to ileal mucosa (44). Here, we found a similar enhancement of *S. Typhimurium*-induced enteritis by 5 mM NE using this model (Fig. 2). Although it is possible that intestinal damage caused by *S. Typhimurium* might allow NE greater access to adrenergic receptors on the basolateral surface of mucosal cells, intestinal NE has previously been shown to promote fluid uptake from the intestinal lumen (6, 26), so host responses to NE are unlikely to explain the increased fluid secretion observed.

The NE enhancement of *S. Typhimurium*-induced enteritis was not significantly inhibited by deletion of one or both of the *qseC* or *qseE* genes, showing that NE sensing and signal transduction via these receptors is not required for the effect. In *E. coli* O157:H7, QseC and QseE influence expression of flagella and T3SS genes in the presence of NE (39, 42). Such factors play key roles in *S. Typhimurium*-induced enteritis in bovine ileal loops (4, 11, 40, 46, 48). Our in vitro experiments showed that expression and secretion of T3SS proteins were not up-regulated by NE and were not significantly affected by deletion of *qseC* or *qseE*. This is consistent with recent reports on the global transcriptional response of *Salmonella* to epinephrine and analysis of the *qseBC* regulon (25, 31) but differs from other reports (3, 37). Similarly, addition of NE or deletion of *qseC* had no significant effect on motility although deletion of *qseE* or both genes slightly reduced motility. These discrepancies may arise from differences in the strains or mutations used or in the culture medium or assay conditions.

In a mouse model, an *S. Typhimurium* *qseB* mutant showed no virulence defect following oral inoculation while a *qseBC* double mutant killed all mice but showed a consistent delay in mortality (31). This *qseBC* mutant exhibited a slight defect in a coinfection experiment (CIs of 0.344 and 0.326 in spleen and liver, respectively) (31). In a different study, a *qseC* mutant showed delayed virulence for mice following intraperitoneal inoculation (37). In calves we found little or no significant attenuation of a *qseC* or *qseCE* mutant in intestinal colonization or fecal excretion although in many cases the mutants were found in lower numbers than the parent. In contrast, another study showed that a *qseC* mutant exhibited substantially decreased colonization of enteric sites relative to the wild-type in pigs (CIs of 0.024 to 0.067) (3). These differences may reflect the different animal species, strains, routes of infection, and the nature of the mutations.

The NE enhancement of *S. Typhimurium*-mediated enteritis was accompanied by an increase in net bacterial replication. The results for wild-type, $\Delta qseC$, and $\Delta qseCE$ (Fig. 4A) showed that more bacteria were recovered with NE than without. There was a corresponding decrease in the proportion of bacteria containing the temperature-sensitive plasmid (Fig. 4B), which confirmed that this had occurred at body temperature. This result mirrored the catecholamine-induced growth observed in vitro in serum-SAPI medium (Fig. 5), a medium that mimics the nutrient-poor and iron-limited conditions encountered in vivo (17, 29). Exogenous ferric iron elicited an increase in net replication of *S. Typhimurium* in this medium, and, since it is well established that NE promotes the supply of ^{55}Fe from transferrin in this medium (14), enhanced iron supply may explain the NE effect although further studies will be required to establish this. Exogenous iron did not enhance transcription of the *Salmonella* pathogenicity island 1 (SPI-1) reporter in serum-SAPI medium, indicating that iron stimulation of this key enteritis-associated locus may not explain the ability of NE to augment enteritis. It is unclear whether this increase in bacterial numbers was solely responsible for the increased pathology; however, similar differences in the net replication of *Salmonella* serovars Typhimurium and Choleraesuis were previously seen in a 12-h loop model that could be correlated with cytokine synthesis and neutrophil recruitment (34).

It remains to be determined if the levels of NE that stimulated *Salmonella*-induced enteritis in the present study are physiologically relevant in the context of stress. Plasma NE levels are typically in the nM to μM range; however, the mesenteric organs contribute over half of all of the NE released in the body, and concentration gradients will exist toward the epithelium. Quantification of free or tissue-associated NE in the intestines is difficult for reasons we have discussed (44). Social stress is correlated with enhanced fecal excretion of *S. Typhimurium* in piglets (7); however, NE levels in the gut were not determined. Recent studies in our laboratory have indicated that release of endogenous NE by administration of 6-hydroxydopamine (which damages noradrenergic nerve terminals) transiently, but significantly, enhances *S. Typhimurium* excretion when it is given to pigs after oral infection and that it elicited a transient rise in plasma NE levels (data not shown). This lends indirect support to the potential for NE to alter the course of *Salmonella* infection but, again, could not be correlated to NE levels in the intestines.

In conclusion, our data further support the notion that stress-related catecholamines have the potential to enhance the virulence of enteric bacterial pathogens. The effect was associated with increased bacterial replication, consistent with the role of NE in promoting growth under iron-restricted conditions *in vitro*. In contrast, putative adrenergic sensor kinases that have been proposed to be a vital link in interkingdom signaling were not required for NE-induced enteritis, NE-promoted net replication *in vivo*, or catecholamine-stimulated growth *in vitro*. The weak role of the sensor kinases in bovine intestinal colonization suggests that adrenergic receptor inhibitors of the kind described recently (37) are unlikely to be successful for the control of enteric salmonellosis. It remains a possibility that the *Salmonella* QseC and QseE orthologues sense different signals than their counterparts in *E. coli* O157:H7 (they are 88% and 93% similar to their *E. coli* counterparts, respectively). The role of these sensors in virulence (10) and the ability of the LED209 inhibitor of QseC signaling to control *Salmonella* and *Francisella* infections in mice (37) may therefore not reflect interkingdom signaling but, rather, may reflect other roles of these systems.

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